#16

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Rea et al.

Serial No.: 09/666,430

Filed: September 21, 2000

For: DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES

Confirmation No.: 6289

Examiner: G. Ewoldt

Group Art Unit: 1644

Attorney Docket No.: 2183-4205.1US

Declaration Under 37 C.F.R. § 1.132

I, _____, a citizen of the Netherlands, hereby declare and state as follows:

That I received a degree in _____ from _____, in 19___; and a Doctor of Philosophy in _____ from _____, in 19___;

That I am among the joint inventors of the referenced patent application;

That I conducted (or worked directly with) the series of tests related to this Declaration;

That the enclosed summary of the tests as set forth below demonstrate DEX-treated DC can exert a potent immunoregulatory effect on Th1-immunity at two levels: directly through the suppression of proliferation and IFN γ secretion by both naive and memory-type T cells, and

indirectly through the mobilization of IL-10-secreting T cells the presence of which can also suppress proliferation and IFN γ secretion Th1 cells and the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination; and

That Example 1 herein was based on Example 4 of the patent application.

I further hereby declare that the enclosed summary of the tests as set forth below correctly reflect the hereinafter described materials, methods, procedures, and results of those tests.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A: Pretreatment with DEX inhibits the phenotypic change induced by LPS triggering of DC. Immature DC were cultured for 24 hours in the absence or the presence of 10^{-6} M DEX and activated with LPS (10 μg/ml) for 48 hours. Flow cytometric analysis showing the immature D1 cell line (immature DC), the LPS triggered mature DC (LPS) and the DEX pretreated LPS matured DC (DEX-LPS).

FIG. 1B: DEX exposed or control immature DC were left in culture without further treatment or stimulated with LPS. Culture supernatants were harvested 48 hours later and IL-12 secretion was analyzed by ELISA. Data are derived from 3 representative independent experiments.

FIG. 2: Pretreatment with DEX impairs the stimulatory capacities of DCs matured with LPS. Allogeneic mixed lymphocyte culture of BALB/c splenocytes with different numbers of DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS) as compared to untreated immature DCS. The IFN-γ production (B) was measured in supernatants taken after 48 hours and the proliferative response (A) was analyzed at 66 hours. Data are derived from 3 representative independent experiments.

FIG. 3: *In vivo* effects of alloreactive splenocytes by intravenous (iv) injection of immature DCs and treated DCs into allogeneic mice.

A) The proliferation of different numbers of responding splenocytes after *in vitro* restimulation with C57BL/6 splenocytes is shown after no treatment or after treatment with

either immature DC, DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS).

B) shows the IFN-γ production by the responding splenocytes at 48 hours after restimulation.

Data are derived from 2 representative independent experiments.

FIG 4: *In vivo* effects of alloreactive splenocytes by subcutaneous (sc) injection of immature DCs and treated DCs into allogeneic mice.

A) The proliferation of different numbers of responding splenocytes after *in vitro* restimulation with C57BL/6 splenocytes is shown after no treatment or after treatment with either immature DC, DEX treated immature DCs (DEX), mature DCs (LPS), DEX treated mature DCs (DEX-LPS).

B) shows the IFN-γ production by the responding splenocytes at 48 hours after restimulation.

C) shows the number of IL-10 producing cells in case the responding splenocytes were restimulated after 7 days *in vitro* stimulation with C57BL/6 splenocytes. Data are derived from 3 representative independent experiments.

FIG 5A: Graft survival of C57BL/6 (H-2^b) donor skin in untreated BALB/c (H-2^d) mice and in mice receiving 7 days before transplantation a sc injection of 1 x 10^6 mature DC (H-2^b) (LPS) or DEX treated mature DC (DEX-LPS). A significantly prolonged graft survival was found in DEX-LPS treated mice compared to mature DC treated mice (p=0.039).

FIG 5B: Graft survival of DBA/1 (H-2^q) donor skin in untreated BALB/c (H-2^d) mice and in mice receiving 7 days before transplantation a sc injection of 1 x 10^6 mature DC (H-2^b) (LPS) or DEX treated mature DC (DEX-LPS). No significant prolonged graft survival was found in DEX-LPS treated mice compared to mature DC treated mice (p=0.92).

FIG. 7: Dex-treated DC preferentially induce T cells secreting IL-10 instead of IFN γ. Non-adherent PBMC were cultured with either allogeneic CD40-triggered DC or allogeneic DEX-treated CD40-triggered DC. Supernatants were harvested at day 5 and used for measurement of IFN γ and IL-10 by ELISA.

FIG 8: T cells pretreated with modulated DC (DEX-DC) inhibit, in a dose-dependent fashion, the proliferation and cytokine production of alloreactive T cells. Alloreactive T cell

cultures were separately generated through primary stimulation of non-adherent PBMC (donor X) during 10 days with either mature DC or DEX-DC (donor Y), after which viable T cells were isolated and counted. The resulting T cell cultures are designated as T-allo and T-dex. Subsequently, a secondary stimulation of T-allo cells was performed in the presence of mature DC (Donor X) as well as in the presence of titered amounts of T-dex. As a control, secondary stimulation was performed by mixing in titered amounts of T-allo cells. (A) Proliferation was determined after 48 hours of culture by addition of ^{3}H-thymidine for the final 16 hours. (B) Supernatants, harvested from the cultures before addition of ^{3}H-thyxnidine, were used for measurement of IFN-γ production. A representative result of three independent experiments is shown.

EXAMPLE 1

DEK-treated CD40-triggered DC are capable of suppressing Th1-type immunity

Further examination of the T cell responses induced in an allogeneic MLR by CD40-triggered DC versus DEX-treated DC (FIG. 5 of pending application) learned that induction by Dex-treated DC did not merely alter the magnitude of the alloreactive T cell response, but profoundly affected the cytokine production by the T cells stimulated. Whereas the cytokine profile of T cells induced by CD40-triggered DC primarily featured the Th1-type cytokine IFN γ, that of T cells induced by DEX-treated DC was dominated by the immunoregulatory cytokine IL-10 (FIG. 7 attached hereto). This observation prompted us to test whether the T cells induced by DEX-treated DC could themselves exert immunoregulatory properties towards T cell proliferative capacity and IFN γ secretion. Indeed, DEX-DC educated T cells, when mixed in with secondary allogeneic MLR cultures in the presence of CD4-triggered DC, were capable of strongly inhibiting the proliferation and IFN γ production by already primed alloreactive I cells.

Taken together our data demonstrate that DEX-treated DC can exert a potent immunoregulatory effect on Th1-immunity at two levels: directly through the suppression of proliferation and IFN γ secretion by both naive and memory-type T cells, and indirectly through the mobilization of IL-10-secreting T cells the presence of which can also suppress proliferation and IFN γ secretion Th1 cells.

EXAMPLE 2

DEX-treated activated DC suppress anti-transplant immunity in vivo

Treatment of immature DC with an activating trigger in the presence of a glucocorticoid hormone results in DC maturation through an alternative maturation pathway. We demonstrate in a mouse transplantation model that such alternatively matured DC can successfully be exploited for the induction of donor-specific transplantation tolerance *in vivo*.

Materials and Methods

Mice

Female BALB/c (H-2^d), C57BL/6 (B6; H-2^b) and CBA/Ca (H-2^k) mice were obtained from IFFA Credo (Paris, France). B6.C-$H2^{bml}$/ByJ (class I K^b mutant phenotype) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained under specific pathogen-free conditions and used at 6-10 weeks of age.

Cell lines

D1 cell line, a long-term growth-factor dependent immature splenic DC line derived from B6 mice, was cultured as described (Winzler et al, 1997, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures, *J. Exp. Med.* 185: 317). Both floating and adherent D1 cells (detached using 2 mM EDTA) were collected and used.

Treatment of DC

The D1 cells were pretreated with dexamethasone (DEX) 10^{-6} M for 24 hrs, after which LPS or nothing was added to the culture for another 48 hours. D1 treated with LPS only (for 48 hours) were also used. Both DEX and LPS (of *E. coli* (Serotype 026:B6)) were purchased from Sigma-Aldrich. After treatment, supernatants were analyzed for the presence of IL-10 and /or IL-12.

Antibodies and cell surface immuno-fluorescence

The following Abs were purchased from PharMingen: FITC-coupled anti CD86 (B7.2), PE-coupled anti CD80 (B7.1), PB-coupled anti-CD40 and PB coupled anti-I-$A^{b/d}$ (M5/114,

MHC class II). Staining was carried out at 4°C for 30 min. Stained cells were analyzed using a FACScan® flow cytometer equipped with CellQuest software (Becton Dickinson).

Cytokine analysis

Harvested supernatants were tested for IL-12 p40/p70, IL-10 or IFN-γ content using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12 p40/p70 mAb (clone C15.6, PharMingen), rat anti-mouse IL-10 mAb (clone JES5-2A5, PharMingen) or rat anti-mouse IFN-γ mAb (clone R4-6A2, PharMingen). Detection Ab: biotinylated rat anti-mouse IL-12 p40/p70 mAb (clone C17.8, PharMingen), biotinylated rat anti-mouse IL-10 mAb (clone SXC-l, PharMingen) or biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2, PharMingen). Streptavidin-HRP and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively. OD405 was read by an ELISAreader (Wallac, Turku, Finland).

Proliferation assays

To study alloreactivity, splenocytes (0.5 or 1 x 10^5 cells/well) of BALB/c mice were cocultured with irradiated (30 Gy, two-fold dilutions from 2 x 10^4 cells/well) D1 cells or splenocytes (30 Gy, 1 x 10^5 cells/well). Immature DC pretreated with DEX (DEX), DEX-pretreated immature DC subsequently activated with LPS (DEX-LPS) and LPS matured DC (LPS) were used as APC in the stimulation assays. The cells were plated out in U-bottom 96-well plates (Costar, Cambridge, MA, USA) in Iscove's (IMDM, BioWhittaker) containing 8% heat-inactivated Fetal Calf Serum (Greiner, Alphen, The Netherlands), 100 IU/ml penicillin, 2mM L-glutamin and 20 μM 2-ME. Supernatant was harvested after 48 hours and stored at 20°C. Wells were pulsed with 1 μCi ^{3}H-thymidine (Amersham International, Amersham, UK) and the cultures harvested onto glass fiber filters 18 hours later. Proliferation was measured as ^{3}H-thymidine incorporation by liquid scintillation spectroscopy using a betaplate (Wallac, Turku, Finland).

In vivo treatment with modulated DC analyzed by in vitro alloreactivity

After washing, 10^6 D1 cells were injected intravenously (iv) or subcutaneously (sc) in BALB/c mice in PBS with 0.5% BSA. After 7 days, spleen cells were used for detection of

alloreactive cellular responses (proliferation and cytokine analysis) by *in vitro* stimulation with splenocytes from syngeneic mice (BALB/c), from donor mice (C57BL/6) or from third party mice (CBA/Ca).

Elispot analysis

For Elispot analysis 1 x 10^6 splenocytes (from *in vivo* treated mice) were incubated with 1 x 10^6 C57BL/6 splenocytes in a 24-well plate (Costar) in Iscove's containing 8% heat-inactivated Fetal Calf Serum, 100 IU/ml penicillin, 2mM L-glutamin and 20 μM 2-ME days. The cells were harvested and incubated (at either 1 or 2 x 10^5 cells/well) with irradiated (3000 rad) splenocytes (1 x 10^5 cells/well) from C57BL/6, medium or con A controls during 24 hours in a plate (MAHA S45 10, Millipore) that was precoated with 5 μg/ml antibody (IFN-γ: R4-6A2, IL-10: JES5-2A5). Next, the wells were washed and the detection antibody was added at 0.3 μg/ml (IFN-γ: XMG1.2-biotin, IL-10: SXC-1-biotin) and incubated for 2 hours at room temperature. After another washing step, the conjugate (extravidin alkalin phospatase, Sigma E2636) was added and incubated for 1 hour at room temperature. After washing, the substrate was added and incubated for 10 minutes at room temperature, after which the reaction was stopped with tap water. Analysis of spots was performed by using a BioReader 3000 Pro (BioSys, Karben, Germany).

In vivo treatment with modulated DC analyzed by skin transplantation

After washing, 10^6 D1 cells were injected iv or sc in BALB/c mice in PBS with 0.5% BSA. After 7 days, mice were transplanted on the tail with skin grafts derived from the tail from donor mice.

The skin grafts were protected with a glass pipe of 4.5 cm long, which was kept on the tail for 7 days. Beside this protection, little irritation (and therefore inflammation) was observed because of the fact that the mice were kept on individual basis in cages with a high tech artificial bedding (Omega-Dri) instead of normal sawdust. Graft survival was followed by daily visual inspection. Scoring was performed by comparing with syngeneic grafts and was based on redness, crust-forming and the presence of hairs. The grafts were scored as rejected when they were fully necrotic or fallen off. Statistical analysis was performed using the log rank test.

RESULTS

Characteristics of alternatively activated dendritic cells (phenotype and cytokine production)

A typical FACS profile of immature DC and the influence of DEX treatment and LPS triggering on these DCs can be seen in FIG. 1A. DC matured with LPS showed significant up-regulation of CD86, CD40 and MHC class II (middle panel), when compared to immature DC, whereas DC pretreated with DEX and subsequently matured with LPS (DEX-LPS) did not show up-regulation of CD86 and only marginal up-regulation of CD40 and even a lowered expression of MHC class II (lower panel). We investigated whether DEX affected the production of the pro-inflammatory cytokine IL-12. As shown in FIG. 1B, LPS triggering of immature DC strongly induced IL-12 (p40/p70) secretion. Combined treatment with DEX and LPS resulted in a strongly reduced (7-fold) IL-12 production compared to LPS treatment alone, whereas DEX treatment only also resulted in a dramatically reduced IL-12 production.

Impaired stimulating capacity of alternatively activated DC

The reduced IL-12 production by DEX treated LPS triggered DC (DEX-LPS) prompted us to assess the T cell stimulatory capacity of these DC. As shown in FIG. 2A, proliferation of BALB/c (H-2^d) splenocytes in a primary MLR response to stimulation with B6 derived (H-2^b) DEX-LPS DC was strongly reduced (and similar to the allogeneic response to untreated immature B6 DC). Similar striking differences of the allogeneic (major and minor histocompatibility antigens mismatched) response were observed when IFN-γ production of the BALB/c splenocytes in response to the various DC used as stimulator cells was measured (FIG. 2B). Besides DEX-LPS DC also the DEX treated immature DC (DEX) induced strongly reduced alloreactive responses as measured by IFN-γ production. Therefore, these results show that mature DC pretreated with DEX have an impaired stimulating capacity.

In vivo reactivity induced by classical versus "alternatively activated" DC

To study the modulation of allo-specific immunity of these DC *in vivo,* these *in vitro* pretreated cells were injected via 2 different routes, either iv or sc. Spleen cells were harvested at different times after injection and restimulated with allogeneic splenocytes *in vitro.* Spleen

cells from mice injected iv with mature DC (LPS), exhibited a high proliferative allogeneic response which was significantly higher than that of untreated control mice or of mice treated with immature DC, DEX treated immature DC (DEX) or DEX-LPS DC (FIG. 3A). Analysis of the production of IFN-γ by the splenocytes of mice injected with the different DC revealed that mice injected iv with the DEX DC showed a similar IFN-γ production as the mice injected with DEX-LPS DC. This response was slightly higher compared to untreated control mice but significantly lower compared to mice injected with untreated immature DC or with LPS DC (FIG. 3B). The proliferative responses of spleen cells from mice injected sc with DEX-LPS DC exhibited a low proliferative allogeneic response which was similar to that of untreated controls and to that of DEX DC (FIG. 4A). The allogeneic IFN-γ response after the DEX-LPS DC treatment was slightly higher or comparable to that of untreated controls, but significantly reduced when compared to mature DC treatment, whereas the DEX DC induced a response similar to mice injected with untreated immature DC (FIG. 4B). The number of IFN-γ producing cells as measured by ELISPOT analysis was 4 times lower after treatment with DEX-LPS DC than after treatment with the DEX DC but comparable to that of untreated controls (data not shown). When the splenocytes were *in vitro* stimulated with C57BL/6 alloantigens for 6 days and restimulated with either conA or C57BL/6 splenocytes, the ELISPOT analysis showed an increase in the number of IL-10 producing cells when compared to untreated or to LPS-DC treated mice (FIG. 4C).

The third-party reactivity was not altered in the DC treated mice compared to untreated mice, indicating that the treatment with alternatively activated H-2^b DC was specific for the H-2^b alloantigens. These experiments demonstrate that DEX-LPS DC induce an alloimmune response, which, based on the *in vitro* parameters tested, showed both quantitative and qualitative differences compared to the alloimmune response found after injection with mature DCs.

Prolonged skin allograft survival after injection with alternatively activated DC

Subsequently, we analyzed the *in vivo* "modulatory" potential of the DEX-LPS DC in a fully allogeneic skin graft model. BALB/c mice were injected sc with either LPS DC or DEX-LPS DC or left untreated. One week after treatment these mice were transplanted with a skin

graft derived from the tail of a donor C57BL/6 mouse. The skins derived from C57BL/6 mice were rejected by the mice injected with LPS DC with a median survival time of 14 days which is not significantly different from the survival in untreated mice (MST 16 days, FIG. 5A). However, when mice were transplanted after injection with DEX-LPS DC, a significantly prolonged allograft survival was found (MST 34 days $p=0.039$). A similar significant prolongation was observed in 2 other independent experiments using BALB/c mice as responding strain ($p=0.023$ and $p=0.009$) and in another study using BM1 mice as responder strain ($p=0.008$, data not shown).

The prolonged skin graft survival after treatment with alternatively activated H-2^b DC was specific for the H-2^b alloantigens as mice injected with DEX-LPS DC rejected skin grafts from DBA/1 mice (H-2^q) in the same time (MST 14 days, FIG. 5B) as control mice (MST 14 days, untreated or LPS DC treated mice $p=0.90$, $p=0.92$ resp). These results show that the DEX-LPS DC are capable of inducing a specific prolongation of complete MHC incompatible skin allograft survival.

DISCUSSION

The present study shows that addition of a glucocorticoid hormone to immature DC results in a decreased proliferative response and a decrease in IFN-γ production by BALB/c splenocytes stimulated by these DCs. In addition we demonstrate that *in vivo* treatment with DEX pretreated mature DC decreased the allogeneic ml response as shown by a reduced IFN-γ production *in vitro* and a reduction in number of IFN-γ producing effector cells when the response was compared to mice pretreated with mature DC. This was the case both after sc or iv injection of the DEX pretreated DCs, but even more after *in vivo* treatment with the alternatively activated (DEX-LPS) DC. Pretreatment of recipients with these DC leads to a significantly prolonged skin graft survival.

In conclusion, our studies confirm and extend the practical use of alternatively activated DC for modulation of the alloimmune response and show that these can induce a prolonged skin graft survival even in a complete MHC incompatible donor-recipient combination.

I hereby declare that all statements made herein of my own knowledge are true and that

all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the U.S. Code and that such willful false statements may jeopardize the validity of the patent.

_____ _____

[name] Date

Residence: _____, The Netherlands

Post Office Address: _____, The Netherlands

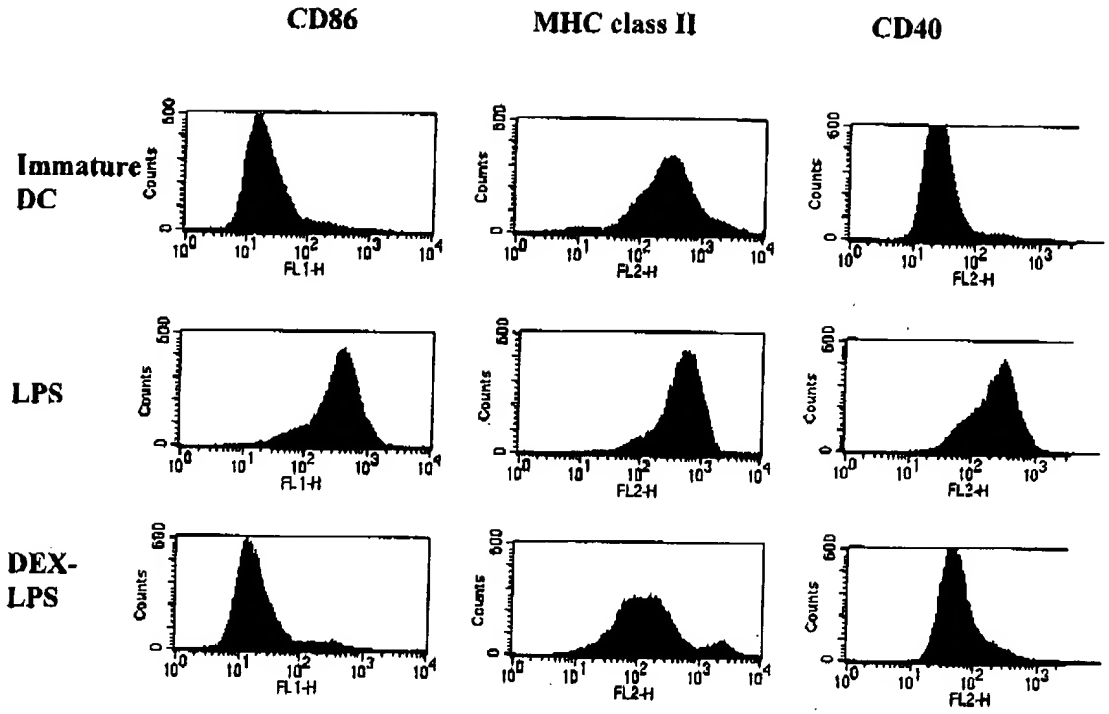
Date: March 18, 2003

KWP/csw

N:\2183\4205.1\Declaration.doc

Figure 1

A)


CD86
MHC class II
CD40
Immature DC
LPS
DEX-LPS
Counts
FL1-H
FL2-H

B)

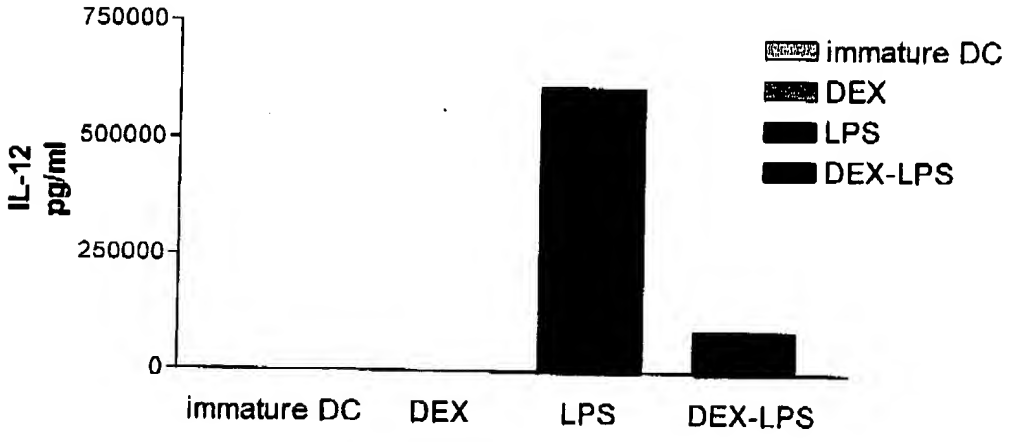
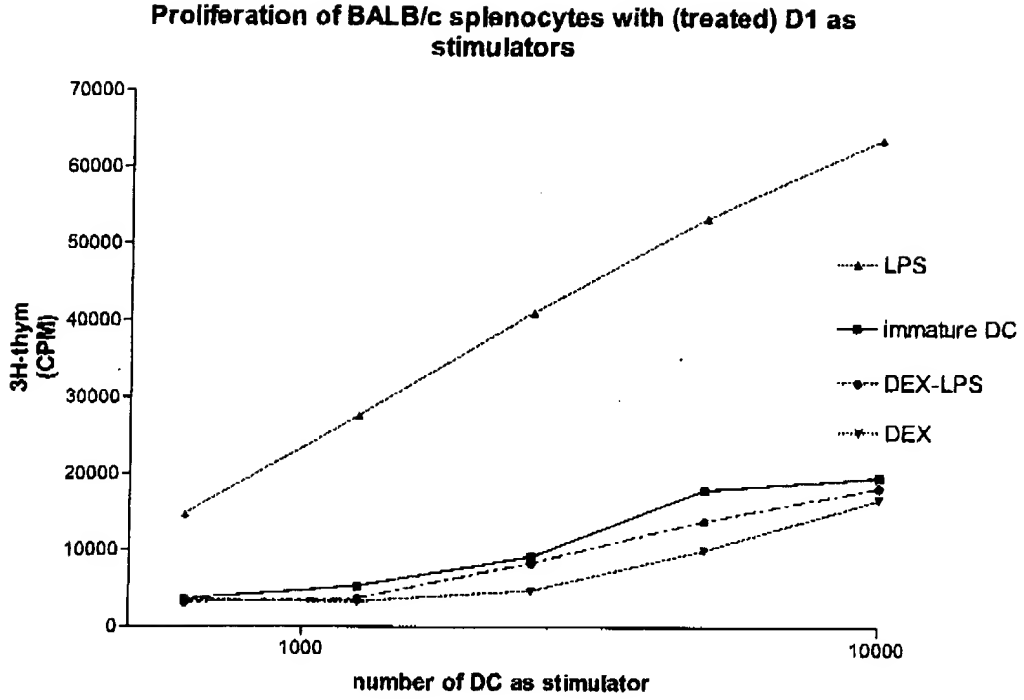

750000
500000
250000
0
IL-12 pg/ml
immature DC
DEX
LPS
DEX-LPS

Figure 2

A)


Proliferation of BALB/c splenocytes with (treated) D1 as stimulators
70000
60000
50000
40000
30000
20000
10000
0
3H-thym (CPM)
1000
10000
number of DC as stimulator
LPS
immature DC
DEX-LPS
DEX

B)

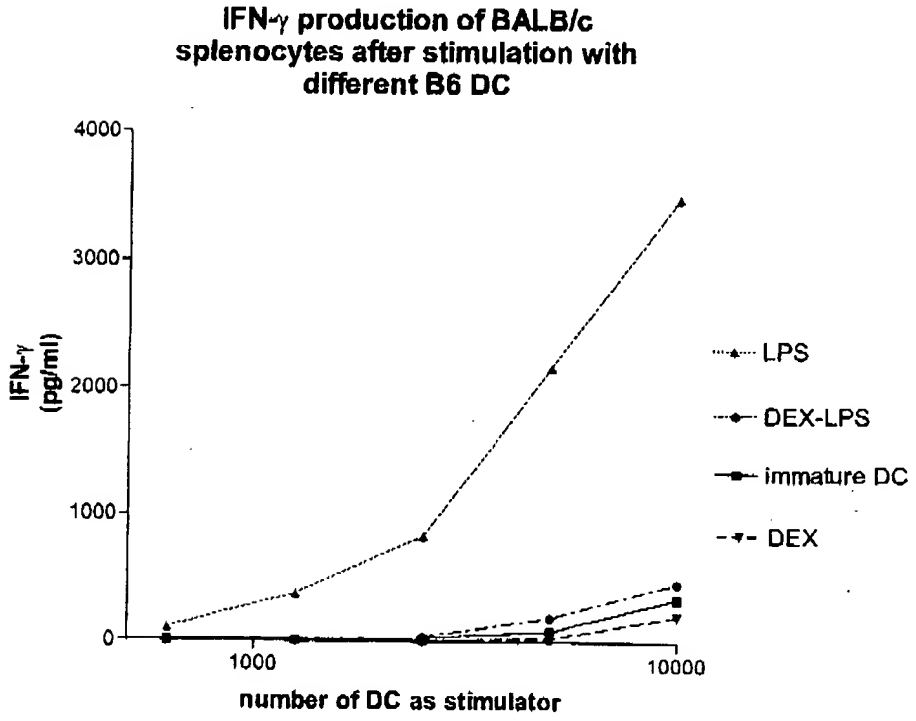
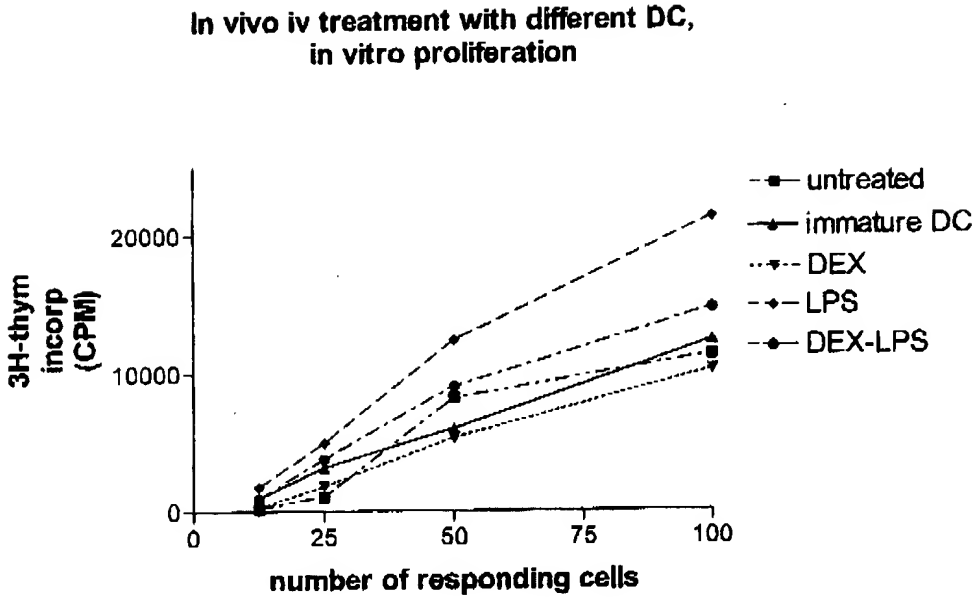

IFN-γ production of BALB/c splenocytes after stimulation with different B6 DC
4000
3000
2000
1000
0
IFN-γ (pg/ml)
1000
10000
number of DC as stimulator
LPS
DEX-LPS
immature DC
DEX

Figure 3

A)


In vivo iv treatment with different DC,
in vitro proliferation
3H-thym incorp (CPM)
20000
10000
0
0
25
50
75
100
number of responding cells
untreated
immature DC
DEX
LPS
DEX-LPS

B)

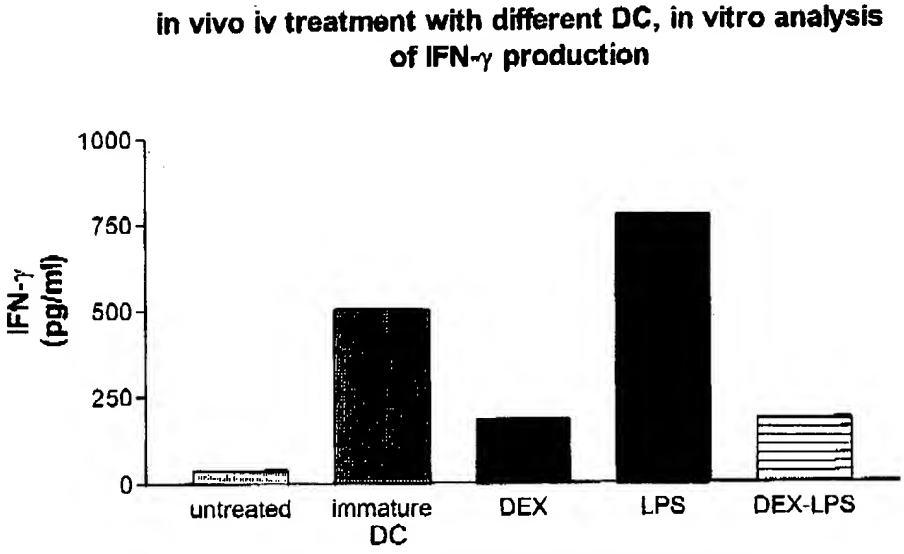
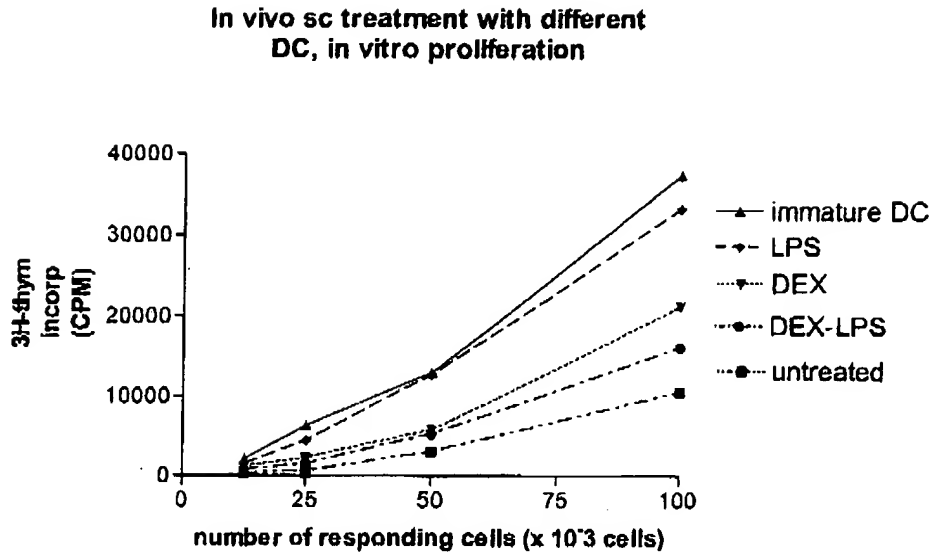
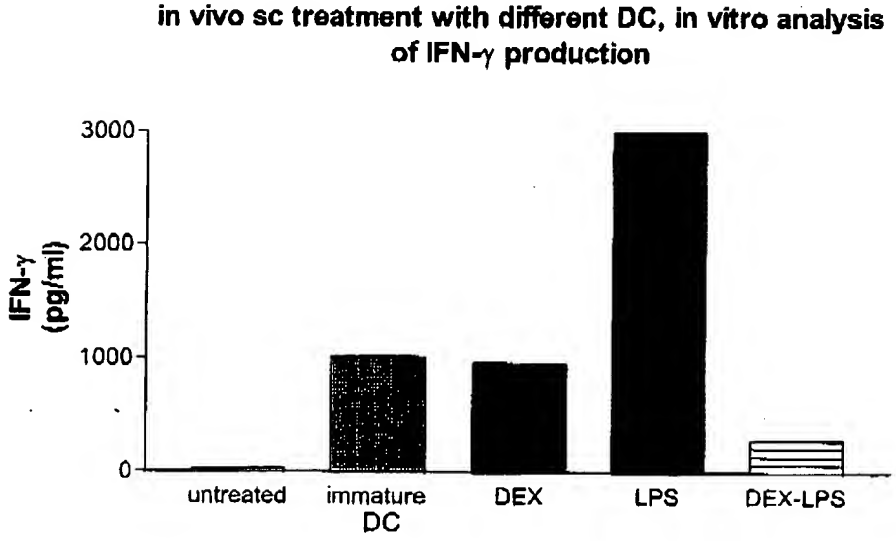

in vivo iv treatment with different DC, in vitro analysis
of IFN-γ production
IFN-γ (pg/ml)
1000
750
500
250
0
untreated
immature DC
DEX
LPS
DEX-LPS

Figure 4

A)


In vivo sc treatment with different DC, in vitro proliferation
40000
30000
20000
10000
0
3H-thym incorp (CPM)
0
25
50
75
100
number of responding cells (x 10^3 cells)
immature DC
LPS
DEX
DEX-LPS
untreated

B)


in vivo sc treatment with different DC, in vitro analysis of IFN-γ production
3000
2000
1000
0
IFN-γ (pg/ml)
untreated
immature DC
DEX
LPS
DEX-LPS

C)

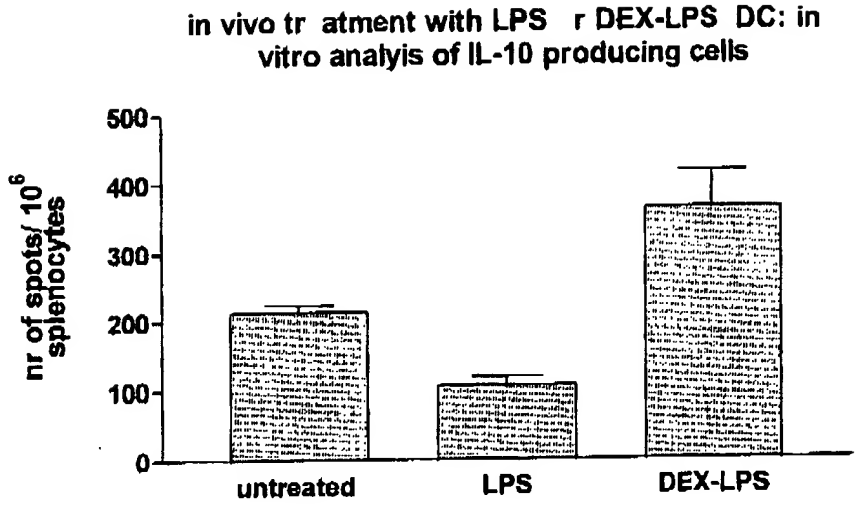
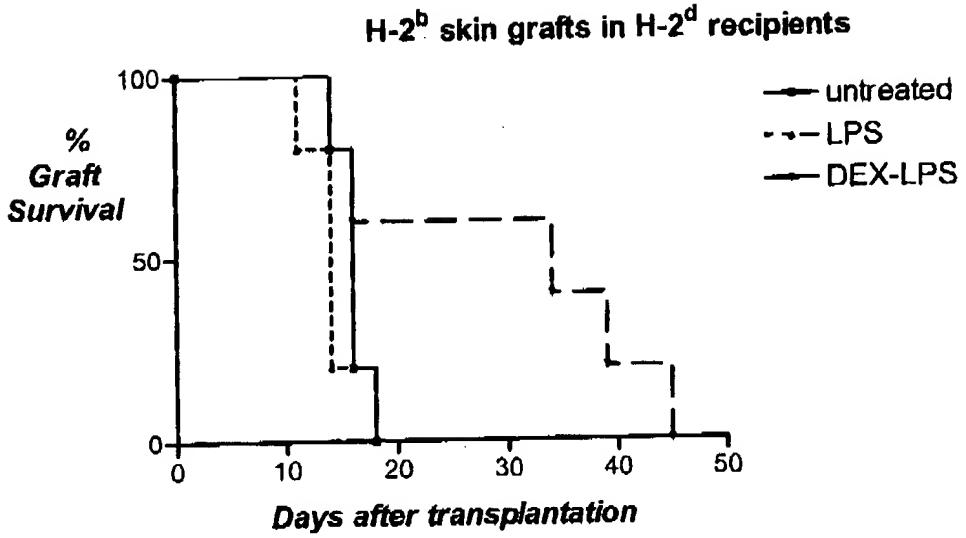

in vivo tr atment with LPS r DEX-LPS DC: in vitro analyis of IL-10 producing cells
nr of spots/ 10^6 splenocytes
500
400
300
200
100
0
untreated
LPS
DEX-LPS

Figure 5

A)


H-2b skin grafts in H-2d recipients
100
50
0
% Graft Survival
0
10
20
30
40
50
Days after transplantation
untreated
LPS
DEX-LPS

B)

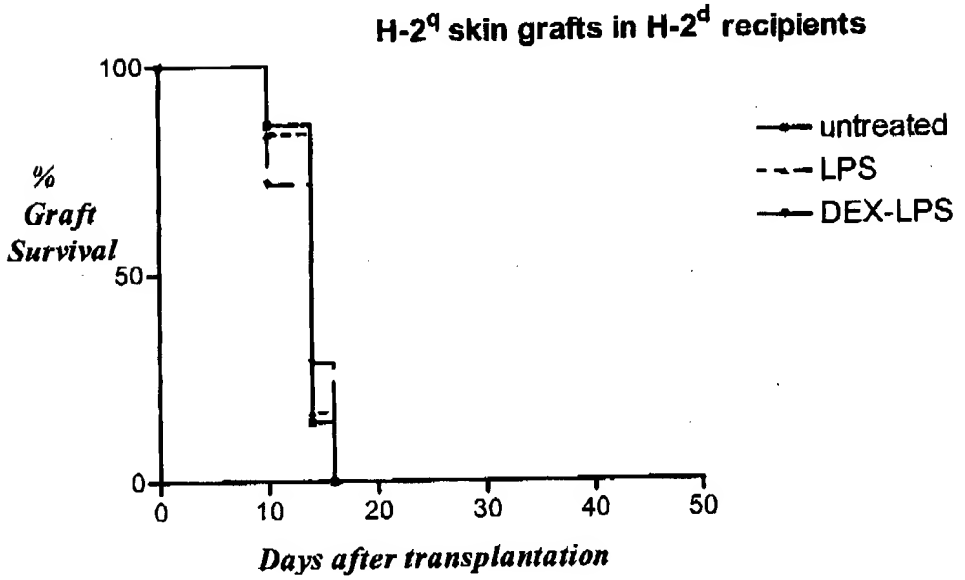

H-2q skin grafts in H-2d recipients
100
50
0
% Graft Survival
0
10
20
30
40
50
Days after transplantation
untreated
LPS
DEX-LPS

Fig. 7: Dex-treated DC preferentially induce T cells secreting IL-10 instead of IFNγ.

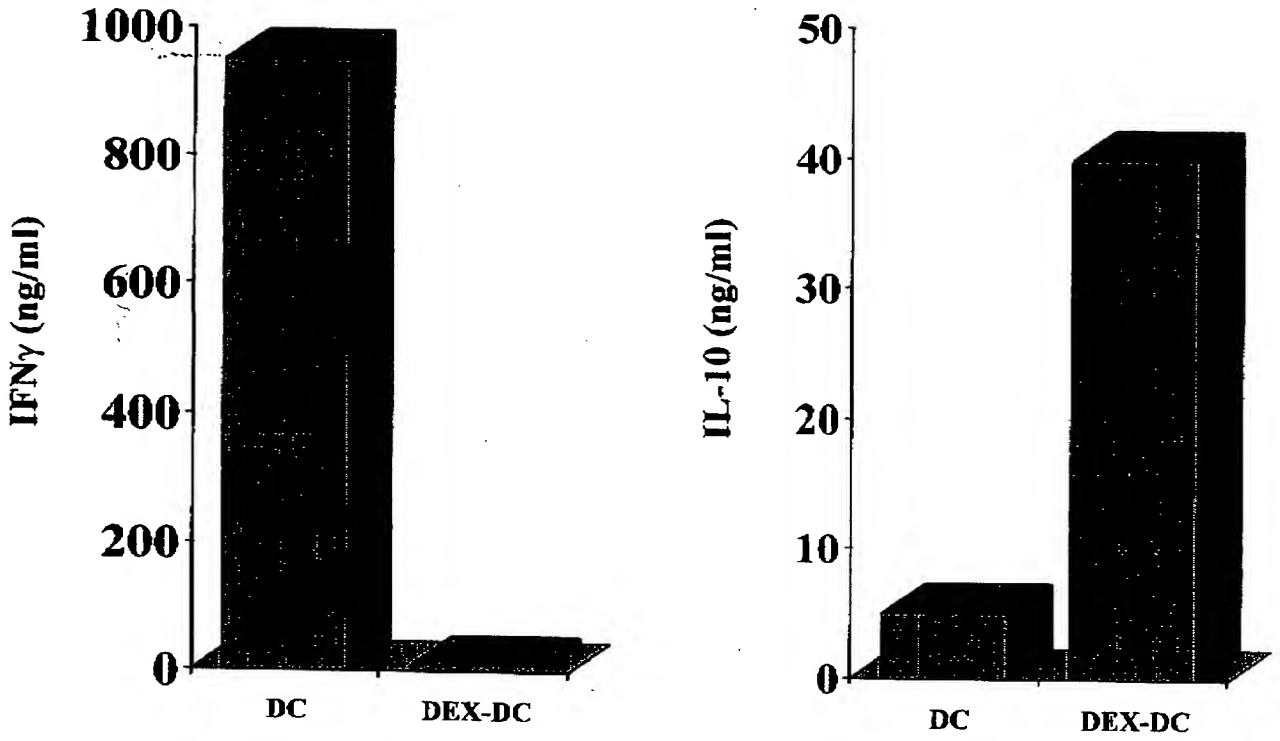


Fig. 8A: DEX-DC educated T cells inhibit secondary alloreactive T cell response

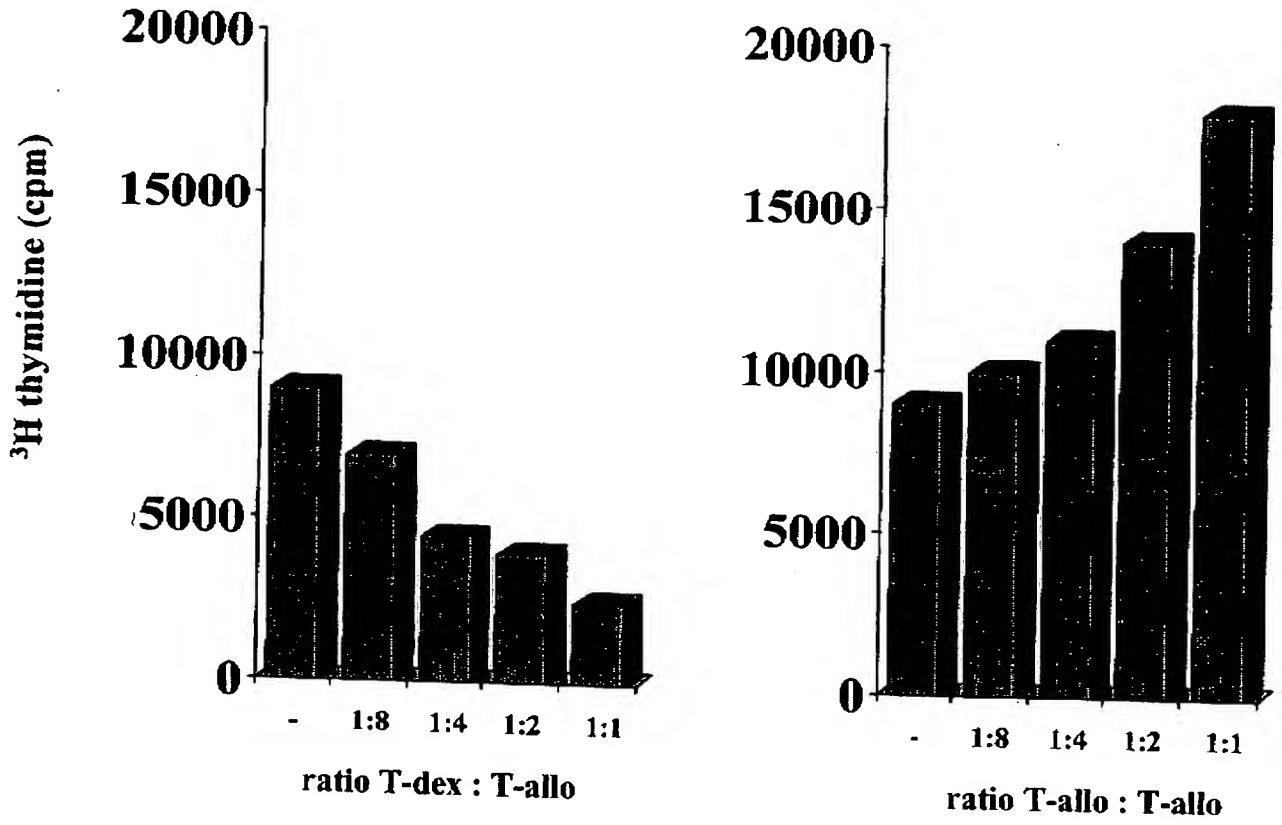
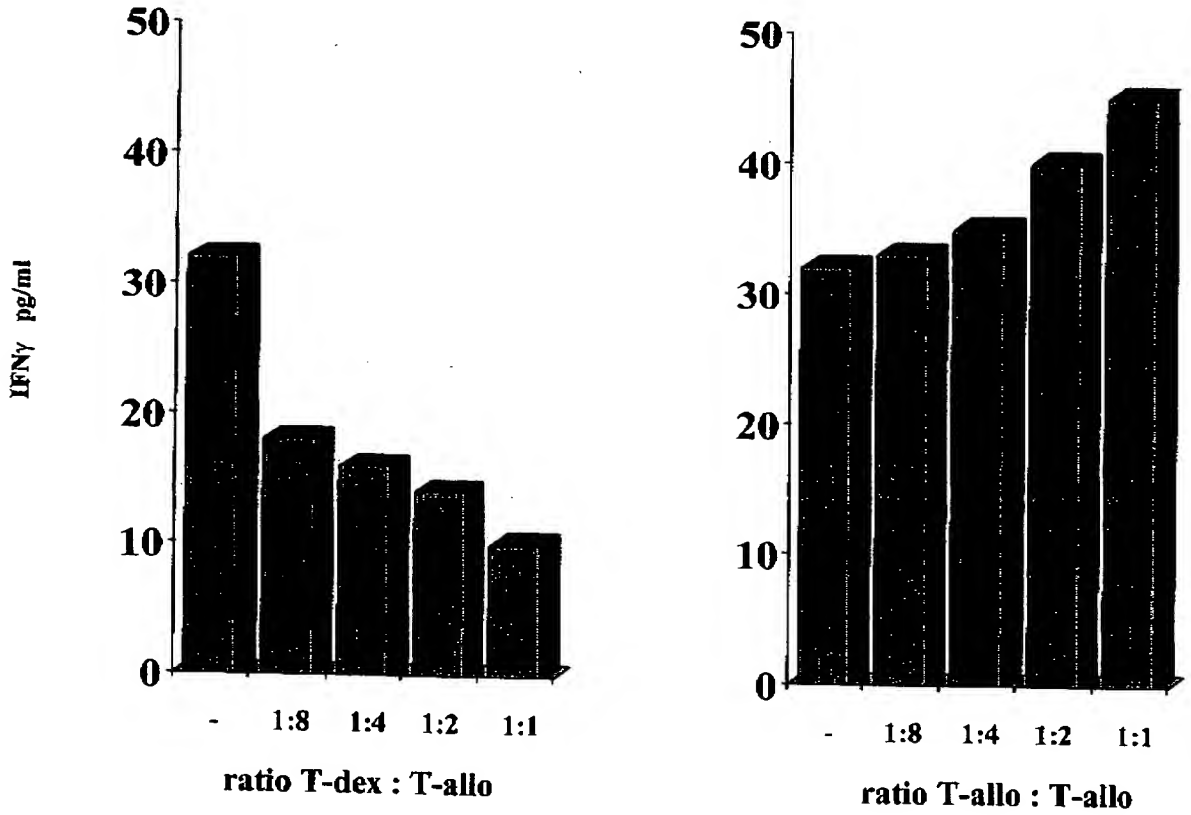


Fig. 8B: DEX-DC educated T cells inhibit secondary alloreactive T cell response



APPENDIX B

(VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS WITH MARKINGS TO SHOW CHANGES MADE)

(Serial No. 09/666,430)

PATENT
Attorney Docket 4205.2US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: _____

Date of Deposit with USPS: _____

Person making Deposit: _____

APPLICATION FOR LETTERS PATENT

for

DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC ~~T CELL~~T-CELL RESPONSES

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TITLE OF THE INVENTION

DENDRITIC CELLS ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC ~~T-CELL~~T-CELL RESPONSES

[0001] Reference to Related Application. This application claims priority from Provisional Application Serial No. 60/157,442, filed October 4, 1999.

[0002] Technical Field. The invention relates to the field of medicine. More in particular, the invention relates to the field of immunotherapy.

BACKGROUND OF THE INVENTION

[0003] The remarkable immunostimulatory properties of dendritic cells ("DC") reside in their ability to transport antigens from peripheral tissues to lymphoid organs where they present these antigens to ~~T cells~~T-cells in an optimal costimulatory context (1). To achieve this complex sequence of events, DC exist in different functional stages. Immature DC behave as sentinels in peripheral tissues where they efficiently capture antigens. Upon pathogen invasion, induction of protective ~~T cell~~T-cell responses ~~requires~~require the activation of immature DC into mature immunostimulatory cells. DC activation is triggered in inflamed tissues by cytokines such as IL-1 and TNF-a and by bacterial components such as lipopolysaccharide (LPS) (2, 3). Activated DC migrate to ~~T cell~~T-cell areas in the lymph nodes while upregulating their costimulatory capacities and optimizing their antigen presenting functions. Upon interaction with antigen-specific T-cells, DC activation is further completed through engagement of the receptor-ligand (1) pair CD40-CD40L, leading to the production of IL-12 (4, 5, 6), a key cytokine for T helper (Th) type 1 and cytotoxic T lymphocyte (CTL) priming (7).

[0004] Antigen Presenting Cell (APC) activation through CD40-CD40L interactions represents an important immunoregulatory step for the establishment of protective ~~T cell~~T-cell immunity against pathogens and tumors (8, 9, 10). This process also plays a key role in the onset of destructive ~~T cell~~T-cell-mediated disorders such as ~~auto-immune~~autoimmune diseases, allograft rejection and graft versus host disease (11, 12, 13). The current treatment of these disorders largely relies on the administration of glucocorticoids (the abbreviation "GC" is used herein for the terms "glucocorticoids" and "glucocorticoid"), which exert potent anti-inflammatory and immunosuppressive effects. Because GC negatively interfere with many aspects of ~~T cell~~T-cell

activation, such as IL-2-driven proliferation and inflammatory cytokine production (reviewed in 14), activated ~~T-cells~~T-cells have long been considered as the main targets for GC action. Several lines of evidence now suggest a role for DC in GC-induced immune suppression. Moser et al. (15) found that GC prevented the spontaneous activation of murine DC thereby decreasing their ~~T-cell~~T-cell stimulatory potential. Kitajima et al. (16) showed that GC could hamper the T-cell-mediated activation of a murine DC line. Viera et al. reported that human DC exposed to GC were poor producers of IL-12 upon LPS stimulation (17). These findings only concern loss of typical DC features and, therefore, favor a simple inhibitory role of GC on DC activation. A more complex immunoregulatory action on the DC system has not been considered.

[0005] The present invention resulted from a detailed analysis of the impact of GC on the CD40-mediated activation of monocyte-derived DC. These DC develop after culture with GM-CSF and IL-4 (2, 18) or after transmigration through endothelial cells (19) and are known to mature into the most potent human Th1-type-inducing APC upon CD40 ligation (5, 20). Moreover, these APC can easily be generated in large numbers and are thereby the cells of choice for DC-based modulation of ~~T-cell~~T-cell immunity (21, 22). In contrast to previous studies, the present invention shows that GC, such as dexamethasone (DEX), do not merely prohibit DC activation, but ~~that it~~ converts CD40 ligation on human monocyte-derived DC and is transformed into an alternative activation pathway. DEX profoundly ~~affect~~affects the CD40-dependent maturation of human monocyte-derived DC, not only by preventing the upregulation of costimulatory, adhesion and MHC surface molecules, but also by causing these cells to secrete the anti-inflammatory mediator IL-10 instead of the Th1 stimulatory cytokine IL-12. In agreement with these phenotypic and functional changes, DC triggered through CD40 in the presence of DEX are poor stimulators of Th1-type responses. Most importantly, the present invention shows that such DC are able to induce a state of hyporesponsiveness in Th1 cells, indicating that these cells are capable of active suppression of Th1-type immunity.

SUMMARY OF THE INVENTION

[0006] As ~~already~~ mentioned above, the impact of GC on DC has been the subject of several previous studies by others. However, in contrast with the present invention, these studies only highlighted inhibitory effects of GC on the DC system. DEX was found to block the upregulation of CD80, CD86 and MHC class II molecules upon activation of murine spleen DC

(15, 16), whereas very recently DEX was demonstrated to also prevent the differentiation of DC from monocyte precursors (28). In these studies, the inability of DC to acquire high expression of costimulatory and MHC molecules was accompanied with a decrease in their ~~T-cell~~T cell stimulatory potential, but the effect of GC on IL-12 production was not investigated. On the other hand, Viera et al found that the effect of GC on LPS-induced DC activation consisted in a 4-fold reduction of IL-12p70 synthesis (17). This partial effect on IL-12 secretion contrasts with the complete suppression of IL-12p70 production which is the subject of the present invention, and can be explained by the fact that their GC-treated immature DC were extensively washed prior to LPS stimulation. We indeed found that upon removal of GC, the effects of these drugs on immature DC were rapidly reversible. The continuous presence of GC during CD40 triggering of DC was clearly preferred in order to stably and completely modulate DC activation (data not shown). Taken together, previous findings indicated that the impact of GC on the DC system should be merely interpreted as an inhibitory event. Importantly, the present invention clearly demonstrates that GC, such as DEX, do not simply suppress DC activation but rather redirect this process towards a distinct functional program.

[0007] DC activation through engagement of CD40-CD40L is a key stimulatory event for the generation of effective Th1 and CD4-dependent CTL responses in vivo (10, 36, 37, 38). This pathway, however, is also involved in the development of unwanted ~~T-cell~~T-cell responses leading to autoimmune disease or organ-transplant rejection (11, 12, 13). Until now, treatment of patients suffering from such disorders largely ~~relies~~relied on the systemic administration of GC hormones. This treatment does not only suppress pathogenic ~~T-cell~~T-cell responses, but also induces a general state of immunosuppression and metabolic and endocrine side effects. The present invention demonstrates that activation of human monocyte-derived DC through CD40, in the presence of GC such as DEX, results in an IL-10-producing APC that is a poor stimulator for Th1-type responses and that can even confer hyporesponsiveness to Th1 cells. The present invention, therefore, indicates that such DC loaded with appropriate antigens can be exploited as a novel approach for specifically downregulating unwanted ~~T-cell~~T-cell responses in vivo.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Fig. 1. Pretreatment with DEX inhibits the phenotypic changes induced by CD40 ligation.

[0009] Seven days immature DC were cultured for 24h in the absence or the presence of 10^{-6}M DEX and activated via CD40 with the CD8-CD40L fusion protein for 48h. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific staining of the indicated cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 4. Data are representative of 4 independent experiments.

[0010] Fig. 2. DC triggered through CD40 maintain an activated phenotype upon a subsequent DEX exposure. Immature DC were activated with the CD8-CD40L fusion protein. DEX (10^{-6}M) or medium control were added 48h later and cells were analyzed after 2 additional days of culture. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific staining of the indicted cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 5. Data are representative of 2 independent experiments.

[0011] Fig. 3. Pretreatment with DEX does not affect the regulation of DC antigen uptake machinery. Immature DC were incubated in the absence or the presence of 10^{-6}M DEX for 24h and further activated or not via CD40 with the CD8-CD40L fusion protein for 48h. Cells were pulsed for 1h with medium containing either 1mg/ml FITC-BSA or 1mg/ml FITC-mannosylated BSA. Empty histograms show the background autofluorescence, Grey-filled histograms show the background uptake at 0°C. and black-filled histograms show the specific uptake at 37°C. Data are representative of 3 independent experiments.

[0012] Fig. 4. Pretreatment with DEX alters the cytokine secretion profile of CD40-triggered DC.

[0013] DEX-exposed or control immature DC were left in culture without further treatment or stimulated with the CD8-CD40L fusion protein. Culture supernatants were harvested 48h later and IL-10, IL-12p40 and IL-12p70 secretion were analyzed by specific ELISA. Data are representative from 6 independent experiments.

[0014] Fig. 5. Pretreatment with DEX impairs the T-cell stimulatory capacities of DC activated via CD40 and leads to a state of hyporesponsiveness of Th1 cells.

[0015] Allogeneic MLR: ~~non-adherent~~nonadherent allogeneic PBMC were cultured with different numbers of CD40-triggered DC, DEX-treated CD40-triggered DC or immature DC. The proliferative response was measured on day 5.

[0016] Th1 stimulation assays: Hsp65-specific T-cells were cultured with different numbers of HLA-DR matched CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the hsp65 protein or with the specific p3-13 peptide epitope. The proliferative response and the T-cell dependent IFN-g production were analyzed on day 3. Data are representative of 4 independent experiments.

[0017] Fig. 6. DEX-treated DC triggered through CD40 induce a state of hyporesponsiveness in Th1 cells. Hsp65-specific T-cells precultured with CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the p3-13 peptide epitope were harvested after 48h, allowed to rest in the presence of 5U/ml IL2 for 3 days, and restimulated with p3-13-pulsed DC. The proliferative response and IFN-g production were measured on day 3. Similar results were obtained in 2 independent experiments.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0018] The den~~t~~dritic cells of the invention ~~posses~~possess different capabilities than those previously reported for dendritic cells. One can, therefore, consider these cells to be part of a class of cells distinct from the class formed by the "classical" dendritic cells. The dendritic cells of the invention can be used in a different way than the classical dendritic cells. The dendritic cells of the invention can, for instance, be used to suppress, at least in part, an undesired immune response in a host. In one aspect, the invention, therefore, provides a method for preparing a pharmaceutical composition for reducing an unwanted ~~T-cell~~T cell response in a host, comprising culturing peripheral blood monocytes from ~~said~~the host to differentiate into dendritic cells, activating said dendritic cells in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cells with an antigen against which ~~said T-cell~~the T-cell response is to be reduced. An unwanted ~~T-cell~~T-cell response can be any type of ~~T-cell~~T-cell response. For instance, but not limited to, a ~~T-cell~~T-cell response associated with an ~~auto-immune~~autoimmune disease or a transplantation disease, such as a graft versus host disease or a host versus graft disease. A pharmaceutical

composition of the invention typically comprises a dendritic cell of the invention suspended in a liquid suitable for preserving the function of ~~said~~the dendritic cell in ~~said~~the liquid and/or suitable for administration to a host. A host, preferably, is a human. Preferably, ~~said~~the host is at risk of developing or is suffering from an ~~auto-immune~~autoimmune disease or allergy. Preferably, ~~said~~the host suffers from or is at risk of suffering from a host versus graft disease and/or a graft versus host disease. With the term "at ~~risk",~~risk," it is meant that one expects that ~~said~~the host may develop ~~said~~the disease, for instance, but not limited to, a host receiving a transplant. Such a host is considered to be at risk of developing a host versus graft disease. An antigen typically is a peptide capable of binding to a major histocompatibility complex (MHC) I and/or II molecule. Such peptides are known in the art and a person skilled in the art is capable of determining whether a given peptide comprises an antigen or not. An antigen may be derived from a naturally occurring protein. An antigen may also be a synthetic peptide or equivalent thereof, preferably with an amino-acid sequence equivalent to a peptide derived from a protein.

[0019] In another aspect, the invention provides a pharmaceutical composition for reducing an unwanted ~~T-cell~~T cell response in a host, ~~said~~the composition being obtained by culturing peripheral blood monocytes from ~~said~~the host to differentiate into dendritic cells, activating ~~said~~the dendritic cells in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cells with an antigen against which ~~said T-cell~~the T-cell response is to be reduced. In one embodiment, a method is provided for reducing an unwanted ~~T-cell~~T-cell response in a host, comprising administering a composition of the invention to ~~said~~the host.

[0020] The invention further provides a method for reducing an unwanted ~~T-cell~~T-cell response in a host comprising culturing peripheral blood monocytes from ~~said~~the host to differentiate into dendritic cells, activating ~~said~~the dendritic cells and/or their precursors in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cells with an antigen against which ~~said T-cell~~the T-cell response is to be reduced and administering ~~said~~the composition to ~~said~~the host.

[0021] In one embodiment of the invention, ~~said~~the activation is done through a CD40 receptor. Activation of DC through triggering of the CD40 receptor can involve either incubation with a CD8-CD40L fusion protein, a trimeric ~~form~~from of CD40L consisting of CD40L-molecules to which a modified leucine zipper has been attached, anti-CD40 antibodies, or cells

that express CD40L. Other signals that can be employed for the activation of DC as described in the present invention include lipopolysaccharide (LPS) and polyI/C.

[0022] In another aspect, the invention provides a method for obtaining ~~an~~a dendritic cell capable of ~~tolerising~~tolerizing a T-cell for an antigen comprising providing ~~said~~the dendritic cell with a glucocorticoid hormone, activating ~~said~~the dendritic cell and providing ~~said~~the dendritic cell with ~~said~~the antigen. With the term ~~tolerising~~"tolerizing," it meant that ~~said~~the dendritic cell has an immunosuppressive effect on ~~said T-cell~~ the T-cell. A ~~tolerised T-cell will essentially~~ tolerized T-cell essentially will not respond with cell division when exposed to a cell presenting an antigen ~~said T-cell would~~, a T-cell in the ~~untolerised~~untolerized state would respond to such exposure with cell division. A ~~tolerised T-cell will essentially~~tolerized T-cell esstentially will not respond ~~with~~by killing a cell presenting an antigen ~~said T-cell would~~, a T-cell in the ~~untolerised~~untolerized state would respond to ~~with cell kill~~such exposure by killing the cell presenting an antigen.

[0023] In one embodiment, ~~said~~the dendritic cell and/or a precursor thereof is provided with ~~said~~a glucocorticoid hormone *in vitro*. A ~~T cell~~T-cell of the invention is preferably an antigen specific ~~T cell~~T-cell, ~~preferably~~ a cytotoxic ~~T cell~~T-cell or a Th cell.

[0024] In another aspect, the invention provides an isolated dendritic cell capable of modifying the function of an antigen specific Th cell, which would otherwise enhance a given immune response, resulting in a ~~T cell~~T cell that is capable of reducing this immune response. In one embodiment, the invention provides a method for modifying an antigen specific T-cell comprising providing an dendritic cell according to the invention with said antigen and ~~co-cultivating~~cocultivating said T-cell and said dendritic cell. Preferably, said ~~co-cultivating~~cocultivating is performed *in vitro*. ~~Said~~The method may further comprise multiplying ~~said~~the functionally modified T-cell.

[0025] The invention also provides an isolated ~~funtionally~~functionally modified T-cell obtainable by a method according to the invention.

[0026] In another aspect, the invention provides the use of a glucocorticoid hormone for obtaining ~~an~~a dendritic cell capable of functionally modifying a T-cell.

[0027] The invention also provides a pharmaceutical composition comprising ~~an~~a dendritic cell and/or a functionally modified T-cell ~~according to the invention~~. The invention further

provides the use of a dendritic cell and/or a functionally modified T-cell ~~according the invention~~ for the preparation of a medicament.

[0028] The invention also provides a method for the treatment of an individual suffering from, or at risk of suffering from, a disease associated with at least part of the immune system of ~~said~~the individual ~~comprising~~, including providing ~~said~~the individual with ~~an~~a dendritic cell and/or a functionally modified T-cell ~~according to the invention~~. Preferably, ~~said~~the dendritic cell and/or ~~said~~the functionally modified T-cell, or precursors thereof are derived from an HLA-matched donor. Preferably, ~~said~~the HLA-matched donor is ~~said~~the individual.

[0029] Method of treatments of the invention are preferably ~~use~~used for the treatment of an individual suffering from an ~~auto-immune~~autoimmune disease, an allergy, a graft versus host disease and/or a host versus graft disease.

Examples

Example 1

Impairment of CD40-CD40L-mediated phenotypic changes by DEX

[0030] We explored the impact of DEX on the phenotypic changes induced by CD40 ligation on immature monocyte-derived DC. In the absence of DEX, the fusion protein CD8-CD40L induced a strong upregulation of the costimulatory molecules CD80, CD86 and CD40, of the MHC class I and II molecules, of the adhesion markers CD54 and CD58 and of the DC maturation marker CD83 (Fig 1). In the presence of DEX, these CD8-CD40L-induced phenotypic changes were dramatically impaired: the upregulation of CD80, CD86, CD40, CD54, CD58 and of the MHC class I and II molecules was largely inhibited and CD83 was not expressed (Fig 1). Importantly, DEX-treated DC did not revert to a monocyte/macrophage stage as shown by the lack of expression of CD14 (Fig 1). Titration of DEX showed a complete inhibition of CD40-mediated phenotypic changes at 10^{-6} M and 10^{-7} M, a partial blockade at 10^{-8} M and no effect at 10^{-9} M and 10^{-10} (data not shown). In addition, DEX action was dependent on binding to the GC-receptor, since it was asbolished by simultaneous addition of the GC receptor antagonist RU486 (data not shown). In experiments performed with LPS or TNF-a as activation agents, similar results were obtained. However, the combination of DEX and TNF-alpha induced a massive cell death (viable cell recovery 5-10% of control cultures), a phenomenon that was not

observed when DEX-treated DC were stimulated with LPS or through CD40 (viable cell recovery 60 to 100% of control cultures) (not shown).

[0031] We next analyzed whether activated DC could still be affected by DEX. DC incubated with CD8-CD40L for 48h and further exposed to DEX maintained a stable activated phenotype (Fig 2).

[0032] We conclude that DEX prevents the phenotypic changes induced by CD40 signals on immature DC and that already activated DC are resistant to DEX action.

Example 2

DEX does not interfere with the regulation of DC antigen uptake machinery

[0033] Unlike activated DC, immature DC efficiently internalize antigens through macropinocytosis and mannose receptor-mediated endocytosis (2, 3, 25, 26). We analyzed whether DEX could affect the DC antigen capture machinery and its downregulation following CD40 cross-linking. As shown in Fig 3, incorporation of FITC-BSA and FITC-mannosylated BSA by immature DC and by DEX-treated immature DC was comparable. Upon CD40 triggering, a similar decrease of FITC-BSA and FITC-mannosylated BSA uptake by both DEX-treated and untreated DC was observed (Fig 3). These results were the first to indicate to us that DEX does not block all aspects of DC activation, since it does not interfere with the down-regulation of the DC antigen capture machinery.

Example 3

DEX-treated CD40-triggered DC secrete IL-10 instead of IL-12

[0034] A key feature of CD40-triggered DC for initiating T-cell immunity resides in their ability to produce the proinflammatory cytokine IL-12 (5, 6, 27). We investigated whether DEX affected IL-12 production by DC stimulated through CD40, and we explored the possibility that DEX could promote the secretion of the anti-inflammatory cytokine IL-10. As shown in Fig 4, CD40 triggering of DC strongly induced IL-12p40 and IL-12p70 secretion (up to 120ng/ml and 170pg/ml, respectively) but only poorly stimulated the production of IL-10 (up to 68pg/ml). In contrast, CD40 triggering of DEX-treated DC resulted in a dramatically reduced IL-12p40 production (up to 100 fold) and in the complete suppression of IL-12p70 secretion, whereas IL-10 production was strongly enhanced (up to 50 fold) (Fig 4). Immature DC and their DEX-

treated counterparts failed to secrete detectable amounts of IL-12 and IL-10 (Fig 4). Therefore, CD40 ligation of DC in the presence of DEX triggers the secretion of high levels of the anti-inflammatory cytokine IL-10 instead of IL-12.

Example 4

DEX-treated CD40-triggered DC are capable of suppressing Th1-type immunity

[0035] The strikingly modified response of DC to CD40 ligation in the presence of DEX prompted us to compare the ~~T-cell~~T-cell stimulatory potential of these cells with that of their DEX-untreated counterparts. In an allogeneic MLR, CD40-triggered DC induced a strong proliferative ~~T-cell~~T-cell response, whereas the addition of DEX prior to CD40 triggering reduced their T-cell stimulatory capacity to that of immature DC (Fig 5). When tested for their ability to stimulate an hsp65-specific $CD4^+$ Th1 clone, CD40-triggered DC pulsed with the hsp65 protein or with the specific peptide epitope p3-13 were found to be potent inducers of both ~~T-cell~~T-cell proliferation and T-cell dependent IFN-g production (Fig 5). In contrast, in the presence of Ag-pulsed DEX-treated CD40-triggered DC, ~~T-cell~~T-cell proliferation and IFN-g production were significantly decreased ($p<0.001$ and $p<0.01$ respectively) (Fig 5). We next investigated whether DEX-treated CD40-triggered DC were simply poor stimulators of Th1 cells, or whether they could exert suppressive effects on these ~~T-cells~~T-cells. We, therefore, tested hsp65-specific T-cells stimulated with p3-13-pulsed DEX-treated CD40-triggered DC for their capacity to respond to a second potent antigenic challenge. Fig 6 shows that preculturing ~~T-cells~~T cells with CD40-triggered DC led to a strong ~~T-cell~~T cell proliferation and IFN-gamma production upon second antigen-specific restimulation. In contrast, preculture with DEX-treated CD40-triggered DC resulted in a dramatically reduced proliferative and IFN-gamma production capacity of Th1 cells. Thus, CD40 triggering of DC in the presence of DEX results in APC that are not merely poor inducers of ~~T-cell~~T-cell responses but that also induce a state of hyporesponsiveness in Th1 cells.

Materials and Methods

Generation of DC

[0036] Immature DC were generated from peripheral blood monocyte precursors (PBMC). Human PBMC from healthy donors, isolated through Ficoll-Hypaque density

centrifugation were plated at $1.5x10^7$ per well in 6-well plates (Costar Corp., Cambridge, MA) in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 2mM glutamine, 100UI/ml penicillin and 10% FCS. After 2h at ~~37°C~~37°C, the ~~non-adherent~~nonadherent cells were removed and the adherent cells were cultured in medium containing 500U/ml IL-4 (Pepro Tech Inc. Rocky Hill, NJ) and 800U/ml GM-CSF (kindly provided by Dr S. Osanto, LUMC, Leiden, NL) for a total of 7 days.

Activation of immature DC with a CD8-CD40L fusion protein

[0037] Activation of DC though CD40 was performed with a fusion protein made of the extracellular domain of human CD40L and of the murine CD8a chain (CD8-CD40L). The CD8-CD40L cDNA described by Garrone et al. (23) was transferred into an eukaryotic expression vector containing the hygromycin resistance gene, and used for the generation of stably transfected Chinese Hamster Ovary (CHO) cells. Culture supernatants containing the CD8-CD40L fusion protein were concentrated with a pressurized stirred cell system (Amicon, Inc., Beverly, MA), checked for binding to CD40 and tested for optimal DC activation conditions (not shown). DC were incubated at 5×10^5/ml/well in a 24-well plate (Costar Corp., Cambridge, MA) and activated in the presence of 1/10 CD8-CD40L supernatant. Cells and supernatants were analyzed after 48h. Of note, control supernatants obtained from untransfected CHO cells or from CHO cells transfected with the CD8a cDNA lacked DC activating functions and were similar to culture medium.

DEX and RU486 treatment of DC

[0038] Seven days immature DC were treated with 10^{-6}M DEX (Sigma, St Louis, MO) in the presence of GM-CSF and IL-4 or GM-CSF alone. After 24h, DC were analyzed or were further stimulated via CD40 by adding the CD8-CD40L fusion protein to the cultures as described above. In some experiments, the glucocorticoid receptor antagonist RU485 (Roussel-UCLAF, Romainville, France) was used at 10mM final concentration, alone or in combination with DEX.

Analysis of DC surface phenotype by flow cyttometry

[0039] Cells were stained on ice with FITC or PE-conjugated mouse monoclonal antibodies (MoAb) for 30 min in PBS 1% FCS and were analyzed on a FACScan® (Becton Dickinson, San Jose, CA). The following MoAb were used: FITC-anti-CD80 (BB1), PE-anti-CD86 (FUN-1), FITC-anti-CD40 (5C3), PE-anti-CD54 (HA 58) and PE-anti-CD58 (1C3) (Pharmingen, San Diego, CA)~~,~~; PE-anti-CD14 (L243) and PE-anti-HLA-DR (Mf-P9) (Becton Dickinson)~~,~~; PE-anti-CD83 (HB15A) (Immunotech, Marseille, France); and PE-anti-HLA class I (Tu 149) (Caltag Laboratories, Burlingame, CA).

Antigen uptake experiments

[0040] DC were resuspended in medium buffered with 25mM Hepes. FITC-BSA and FITC-mannosylated BSA (both from Sigma) were added at 1mg/ml final concentration and the cells were incubated at ~~37°C~~37°C or at ~~0°C~~0°C to determine background uptake. After 1h, DC were washed extensively with ~~iced-cold~~ice-cold PBS and analyzed by FACS® using propidium iodide to eliminate dead cells.

Cytokine detection by ELISA

[0041] Culture supernatants were analyzed in serial ~~twofold~~two-fold dilutions in duplicate. IL-12p70 was detected using a solid phase sandwich ELISA kit (Diaclone Research, Besancon, France) (sensitivity 3pg/ml). For IL-12p40 and IFN-g detection, capture MoAb and polyclonal biotinylated detection Ab were obtained from Peter van de Meijde (BPRC, Rijswijk, NL) (sensitivity 10pg/ml). IL-10 was detected using the Pelikine compact human IL-10 ELISA kit (CLB, Amsterdam, NL) (sensitivity 3pg/ml).

Allogeneic mixed lymphocyte reaction (MLR)

[0042] ~~Non-adherent~~Nonadherent allogeneic adult PBMC from an unrelated individual were cultured in 96-well flat-bottom plates (Costar Corp., Cambridge, MA) at a density of 1.5 x 10^5/well with various numbers of g-irradiated (3,000 rads) DC, in ~~triplicates~~triplicates. Proliferation was assessed on day 5 by [^{3}H]thymidine uptake (0.5mCi/well, specific activity 5Ci/mMol, Amersham Life Science, Buckinghamshire, UK) during a 16h pulse.

Th1 stimulation assays

[0043] The Mycobacterium tuberculosis and ~~M. leprae~~*M. leprae* hsp65-specific, HLA-DR3-restricted CD4+ Th1 clone Rp15 1-1 used in this study recognizes an hsp65 determinant corresponding to peptide residues 3 to 13 (p3-13) (24). HLA-DR-matched DEX-treated immature DC and their DEX-untreated counterparts were pulsed with 10mg/ml of p3-13 or with 10mg/ml of hsp65 for 2h, washed extensively and stimulated through CD40 as described above. For Ag-pulsed DEX-treated immature DC, CD40 triggering was performed in the presence of DEX. Hsp65 specific ~~T cells~~T-cells (10^4) were cultured with different numbers of g-irradiated (3,000 rads) DC in 96-well flat-bottom plates (Costar Corp.) in ~~triplicates~~triplicate for 3 days. [^{3}H]thymidine (incorporation) was measured on day 3 after a 16h pulse. Before the addition of [^{3}H]thymidine, 50ml of supernatants were collected from each well and supernatants from triplicate wells were pooled to measure IFN-g production. To test hsp65-specific ~~T cells~~T-cells responsiveness to a second potent antigenic challenge, 10^4 ~~T cells~~T cells were first cultured for 48h with 5 x 10^3 peptide-pulsed DC prepared as above, then harvested and allowed to rest in medium containing 5U/ml IL-2. Three days later, 10^4 viable ~~T cells~~T-cells were restimulated with 5 x 10^3 peptide-pulsed DC generated from the same donor as used for the first culture and tested for their ability to proliferate and to produce IFN-g as previously described.

Statistical analysis

[0044] Covariance analysis was used to compare ~~T cell~~T cell proliferation and IFN-g production as a function of DC number, between DEX-treated CD40-triggered DC and DEX-untreated CD40-triggered DC (Fig. 5).

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ABSTRACT OF THE DISCLOSURE

The present invention provides novel methods for immunotherapy. The invention provides immune cells and methods to generate them, with the capacity to at least in part reduce an immune response in a host. In one aspect, the invention provides a method for generating a dendritic cell with the capacity to ~~tolerise~~tolerize a ~~T cell~~T-cell for antigen ~~said T cell~~the T-cell was specific for~~, comprising~~including culturing peripheral blood monocytes from an individual to differentiate into dendritic cells, activating ~~said~~the dendritic cells in the presence of a glucocorticoid hormone and loading ~~said~~the activated dendritic cell with ~~said~~the antigen ~~said T cell~~the T-cell was specific for.

Addendum

1. DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES